Mechanisms of Endothelial Hemoglobin Toxicity

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Zusammenfassung

Während hämolytischer Prozesse wird Hämoglobin (Hb) aus den Erythrozyten freigesetzt. Extrazelluläres Hb führt zu Kreislaufinstabilität weil es Signaltransduktionsmoleküle wie Stickstoffmonoxid (NO) abbauen kann. Zusätzlich schädigt freies Hb das Gewebe durch Oxidation, besonders das Endothel. Der Mechanismus dieser Schädigung war bisher weitgehend unbekannt. Mit den vorliegenden Arbeiten haben wir Lipidoxidationsprodukte als die ultimativ toxischen Reaktionsprodukte identifiziert. Freies Hb, speziell Methämoglobin (Fe^{3+}), hat ein nur lose gebundenes Häm-Molekül in der dafür vorgesehenen Bindungstasche. Daher kann dieses Häm leicht verloren gehen und dann in einer apolaren Umgebung akkumulieren, so zum Beispiel in Lipoproteinen oder Zellmemebranen. Darin kann Häm die Lipidperoxidation unter Bildung von Lipidoxidationsprodukten exponentiell steigern, was schlussendlich zur Toxizität auf die Endothelzellen führt. Wir haben Haptoglobin (Hp) als potenten Inhibitor der Hb Toxizität identifiziert. Hp bindet irreversibel an Hb und bildet einen stabilisierenden Komplex, welcher den Hämverlust zuverlässig verhindert. Ferner haben wir mit Hämopexin (Hx) einen zweiten Inhibitor der Hb-Toxizität gefunden. Im Gegensatz zu Hp bindet Hx jedoch nicht an Hb, sondern fängt freies Häm mit sehr hoher Affinität ein und verhindert dadurch den Hämtransfer in die Liposomen. Weitere in-vivo und später klinische Studien sind notwendig, um die protektive Potenz von Hp und Hx basierend auf diesen fundamentalen biochemischen und zellbiologischen Prozessen zu bestätigen.

Abstract

Hemoglobin (Hb) is released from red blood cells during hemolysis. Extracellular Hb causes vascular instability due to depletion of small signal transduction molecules such as nitric oxide. Free Hb also leads to oxidative tissue damage especially on the endothelium: However, the mechanism of this damage was poorly characterized. In the present work, we identified lipid oxidation products as the ultimate toxic reaction species. In free Hb, especially in its ferric redox state (Fe^{3+}), heme is only loosely bound in the heme pockets and thus can easily be released. Free heme accumulates in an apolar milieu such as in liposomes or cell membranes. Inside liposomes, heme is able to exponentially accelerate lipid peroxidation and thus formation of lipid oxidation products, finally exerting endothelial toxicity. We have identified haptoglobin (Hp) as a potent inhibitor of Hb toxicity. Hp binds Hb irreversibly and thus forms a stabilizing complex that completely inhibits heme release. We have further found Hemopexin (Hx) as a second potent inhibitor of Hb oxidative toxicity. Hx, in contrast to Hp, does not bind to Hb but encapsulates free heme at very high affinity and thus prevents translocation of Hx into liposomes. The specific target-specificities of Hp and Hx, respectively, provide a biochemical explanation for the enigmatic description of Hp as the primary and Hx as the backup protection system against Hb toxicity. Subsequent in-vivo and later clinical studies are necessary to confirm the protective potency of Hp and Hx with further data based on the fundamental biochemical and cell biological mechanics identified within this thesis.

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B. Authorship contribution 17

The following publications are part of this cumulative PhD-Thesis:

1. Lipiski M*, Deuel JW*, Baek JH, Engelsberger WR, Buehler PW and Schaer DJ.
Human Hp1-1 and Hp2-2 Phenotype-Specific Haptoglobin Therapeutics Are Both Effective In Vitro and in Guinea Pigs to Attenuate Hemoglobin Toxicity.
Antioxidants & Redox Signaling 2013; 19(14): 1619-33
* these authors contributed equally to this work 20

1. Schaer CA*, Deuel JW*, Bittermann AG, Rubio IG, Schoedon G, Spahn DR, Wepf RA, Vallelian F and Schaer DJ.
Mechanisms of haptoglobin protection against hemoglobin peroxidation triggered endothelial damage.
Cell Death and Differentiation 2013; 20(11): 1569-79
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2. Vallelian F, Deuel JW, Opitz L, Schaer CA, Puglia M, Lönn M, Engelsberger W, Schauer S, Karnaukhova E, Spahn DR, Stocker R, Buehler PW and Schaer DJ.
Proteasome inhibition and oxidative reactions disrupt cellular homeostasis during heme stress.
Cell Death and Differentiation 2013; 20(11): 1569-79 56

3. Deuel JW, Vallelian F, Schaer CA, Puglia M, Buehler PW and Schaer DJ.
Different target specificities of haptoglobin and hemopexin define their sequential role as primary and backup protective system against hemoglobin toxicity. 72
Free Radic Biol Med 2015; 89: 931-43.

Introduction

Hemoglobin (Hb) is released from red blood cells during multiple disease states, including hereditary hemolytic anemia (e.g. hereditary spherocytosis, sickle cell disease), acquired hemolytic anemia (e.g. autoimmune hemolytic anemia, paroxysmal nocturnal hemoglobinuria), infection triggered hemolysis (malaria, hemolytic uremic syndrome and also generally during sepsis), mechanical red blood cell destruction (e.g. artificial heart valves, extracorporeal circulation) as well as during massive blood transfusion (Figure 1). In these conditions, extracellular Hb is both a disease marker and a toxin[5]. Hemolysis can also occur in anatomically defined microscopic regions such as sites of tissue injury, inflammation or within atherosclerotic plaques. Depending on the scale and rate of the hemolytic process, the primary adverse effects that occur during acute and chronic Hb exposure are vascular dysfunction, oxidative tissue damage and deregulated inflammation[6]. These effects can lead to systemic manifestations such as as pulmonary arterial hypertension, renal failure, neuro- and cardiac toxicity (including congestive cardiac failure and acute chest syndrome)[7] but also to more localized processes such as progression and destabilization of atherosclerotic plaques.

Several pathways of Hb mediated endothelial toxicity have been identified: Oxidation of

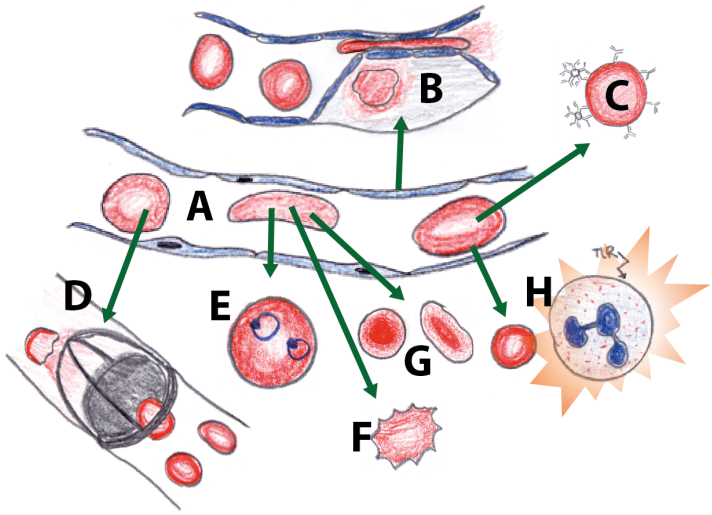

Figure 1: Causes of hemolysis. (A) Under physiologic conditions Hb is concealed within erythrocytes. (B) With atherosclerosis, hemolysis can occur inside the plaque as well as in the now narrowed lumen. (C) During autoimmune hemolytic anemia Hb are opsonized by anti-erythrocytic autoantibodies and then destroyed by complement activation. (D) artificial heart valves and other mechanical devices in the blood stream cause continuous hemolysis by shearing erythrocytes beyond their resistance. (E) intraerythrocytic parasites such as plasmodia cause hemolysis by altering the intracellular homeostasis. (F) overaged blood transfusion as well as (G) erythrocytopathies such as hereditary spherocytosis or ellipsocytosis cause intravascular hemolysis by altering membrane flexibility and thus diminishing erythrocytic elasticity. (H) During inflammation, activated immune cells cause direct hemolysis by producing reactive oxygen species.

ferrous Hb to metHb (Fe^{2+} → Fe^{3+} + e^-), either by autoxidation or driven by external oxidants such as hydrogen peroxide (H_2O_2) or lipid peroxide radicals ($LOO^{\bullet}$) causes heme-globin binding to drastically decline, leading to externalization of free heme. Free heme is cytotoxic by causing oxidative damage to cellular proteins and lipids and impairs cellular homeostasis of damaged proteins by interfering with proteasomal activity. Externalized heme, due to its lipophilic nature, will compartmentalize into the oily phase such as into lipids where it starts a radical driven oxidation of unsaturated fatty acids eventually leading to formation of cytotoxic metabolites. If metHb is further oxidized by external oxidants, it reaches the ferryl-Hb oxidation state (Fe^{3+} + $^{\bullet}O^-$ → $Fe^{4+}=O^{2-}$ + $Porphyrin^{\bullet+}$ + e^-), a Hb species with so high

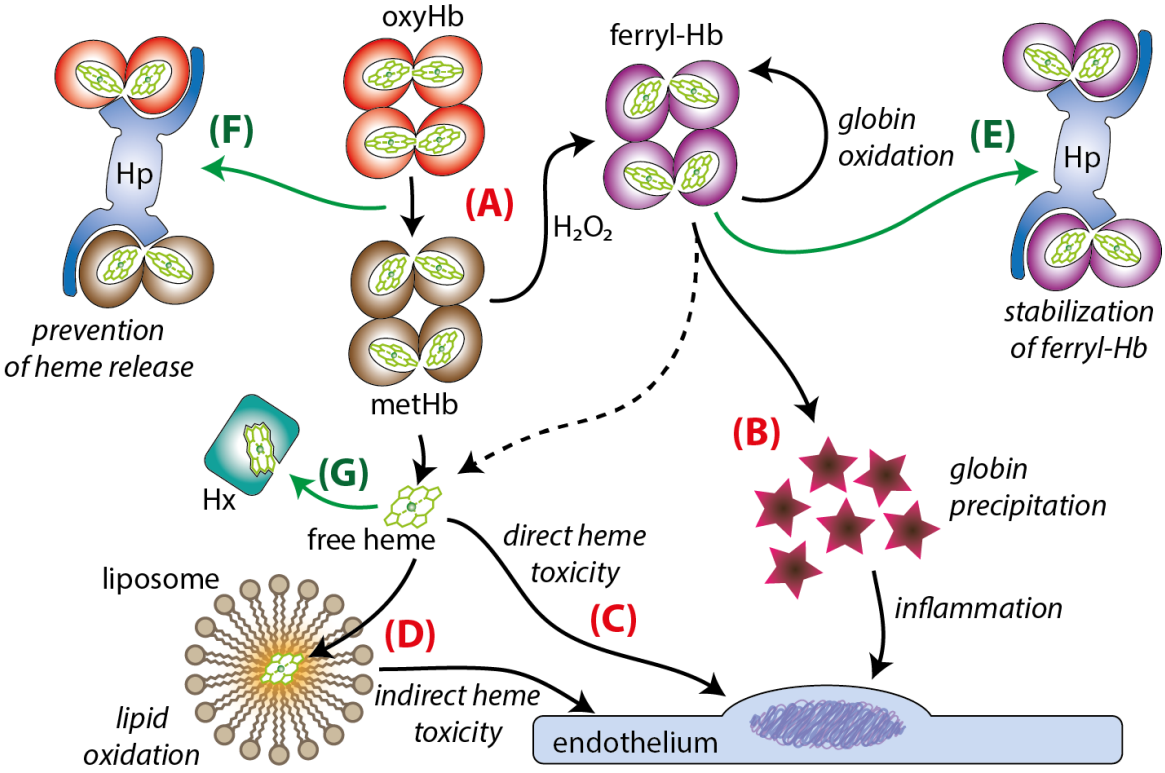

Figure 2: **Attenuation of Hb toxicity by the plasma proteins Haptoglobin (Hp) and Hemopexin (Hx).** (A) OxyHb undergoes auto-oxidation to metHb. In the presence of oxidative substances such as hydrogen peroxide (H_2O_2), this oxidation is acceleration and the even higher oxidated Hb species ferryl-Hb ($Fe^{4+}=O^{2-}$) is generated. (B) Ferryl-Hb can oxidate its globin chains causing protein denaturation and ultimately globin precipitation alongside with heme release. This globin-precipitate triggers endothelium inflammation and thus disruption of the endothelial monolayer. (C) Even without the presence of oxidative substances, metHb can release its heme due to only loose binding. Free heme has a direct cytotoxic effect by protein oxidation and inhibition of the damaged proteins disposal by inhibiting the proteasome leading to accumulation of oxidized proteins. (D) Free heme also is, due to its hydrophobic character, transferred into liposomes and initiates a oxidative chain reaction involving unsaturated fatty acids, ultimately generating toxic oxidized lipid metabolites. (E) Hp structurally stabilizes Hb in all its oxidation states. Especially ferryl-Hb is stabilized by Hp in such a way, that globin oxidation and subsequent denaturation is completely inhibited. (F) Also Hp binds metHb in such a way, that heme binding is massively improved, preventing the initiation of the vicious lipid peroxidation cycle triggered by released heme. (G) Hx has no direct binding affinity to Hb, however, it has a extremely high binding affinity to free heme, capturing heme before it can transfer into the liposomal compartment thus also preventing lipid peroxidation triggered by heme.

oxidative power, that the porphyrin radical can actually delocalize to aromatic amino acids of the globin chain causing globin oxidation and finally denaturation[22]

Haptoglobin and its phenotypes as scavenger proteins

Haptoglobin (Hp), a natural Hb-binding plasma proteins in mammals, binds extracellular Hb dimers in an irreversible large complex[10] that can be cleared by $CD163^{+}$ macrophages and monocytes[11]. Hp is present in human plasma at high concentration of about 2g/l equaling 80µM of heme binding capacity and binds Hb with very high affinity. Binding of Hb to Hp appers to fundamentally alter its potential to induce pathophysiology by sequestering the Hb:Hp complex within the intravascular compartment and thereby limiting tissue exposure; by stabilizing the redox chemistry and the structure of bound Hb; and by preventing the release of heme when Hb is oxidized to metHb (ferric state, Fe^{3+}). Preclinical studies suggest a strong therapeutic potential for Hp infusion in conditions with enhanced intravascular hemolysis[6,12]. The use of Hp as treatment modality is also supported by clinical experience with human plasma-derived Hp products approved for use in Japan[13].

Hp is composed of two different chains, Hpα and Hpβ. While the Hpβ-chain binds a Hbα-Hbβ-dimer, the Hpα-Chain links the Hp molecule together. There are two different forms of Hpα in humans: Hpα_1 forms one disulfide bond to another Hpα-chain while Hpα_2 can form two disulfide bonds. In case of the phenotype Hp1-1, where in both alleles the Hpα_1-Gene is present, Hp-heterodimers in the form of $(Hp\alpha_1)_2(Hp\beta)_2$ are formed. In case of the phenotype Hp2-2, where in both alleles the Hpα_2-Gene is present, circles of Hp-polymers are formed with varying molecular mass in the form of $(Hp\alpha_2)_n(Hp\beta)_n$ where $n>2$. In the case of Hp1-2, both Hp-genes are present and next to both Hp-forms present in Hp1-1 and Hp2-2 condition also linear Hp-polymers in the form of $(Hp\alpha_1)_2(Hp\alpha_2)_n(Hp\beta)_{n+2}$ where $n>0$ are formed. In our study we showed that Hp1-1 and Hp2-2 are both effective in vivo and in guinea pigs to attenuate hemoglobin toxicity[1]. We found no differences between the two phenotypes in Hb binding and intravascular compartmentalization of Hb in vivo. Both attenuate Hb-induced blood pressure response and renal iron deposition. Also, Hb oxidation was not found to be different between the phenotypes, the most oxidized ferryl (Fe^{4+}) Hb redox state was stabilized, heme was retained within the complex in the ferric (Fe^{3+}) condition and Hb-driven low-density lipoprotein peroxidation was prevented. Thus, we concluded that both Hp phenotypes provide equal biochemical and biological protection from Hb toxicity in vitro and in vivo.

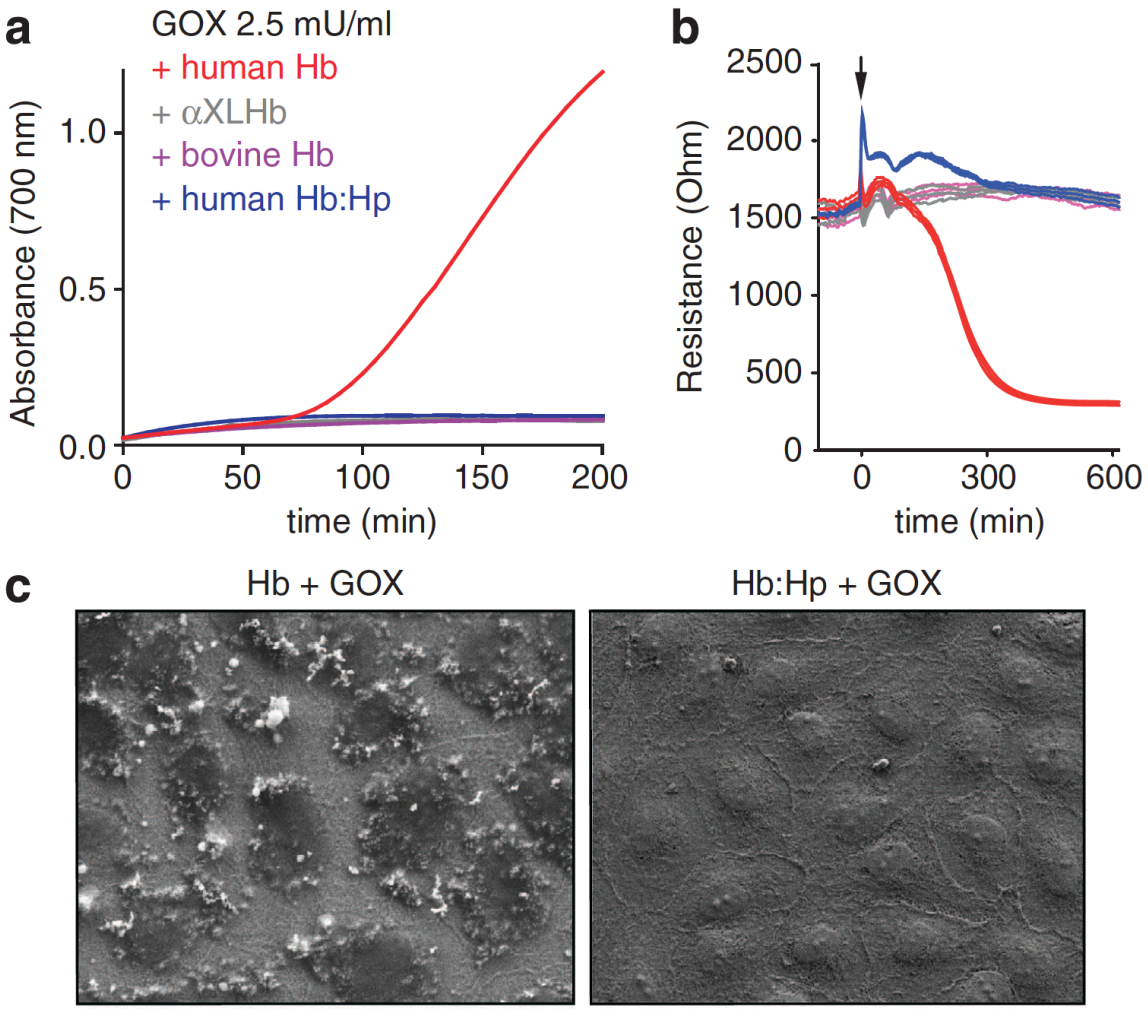

Figure 3: Hb precipitation by oxidation. (a) Aggregate formation during the reaction of hydrogen peroxide generated by glucose oxidate (GOX) 2.5mU/l with Hb at 125µM was recorded by time-resolved spectrophotometry at 700nm under identical conditions as the ECIS-experiment with HPAEC shown in (b). At equal H_2O_2 exposure, endothelial barrier function was compromised in the presence of human Hb bot nut in the presence oft he structure-stable alpha-crosslinked Hb (XLHb) or bovine Hb. The addition of Hp (isostoechiometric) tot he huHb + GOX-Reaction completely prevented aggregate formation and endothelial barrier breakdown. (c) The aggregates/precipitated can be localized on HPAEC by SEM (the images were recorded after 5h) No aggregates can be seen in the presence of Hp. *(This figure is part of publication 2).*

Hb pseudoperoxidase activity

Under peroxidative stress by external hydrogen peroxide we identified globin oxidation and subsequent denaturation as a principal mechanism of oxidative Hb toxicity on endothelial cells. Human Hb is structurally so labile that oxidation by ferryl formation ($Fe^{4+}=O^{2-}$) causes aggregation and precipitation of the globin (Figure 3). We could correlate this precipitate with endothelial inflammation and toxicity[2]. In contrast to human Hb, bovine Hb also reacts with H_2O_2 by ferryl formation, however, no aggregation and no endothelial inflammation or toxicity can be observed. Also structurally stabilized human Hb, covalently cross-linked between the two Hbα-Chains, shows no precipitation and also does not induce the

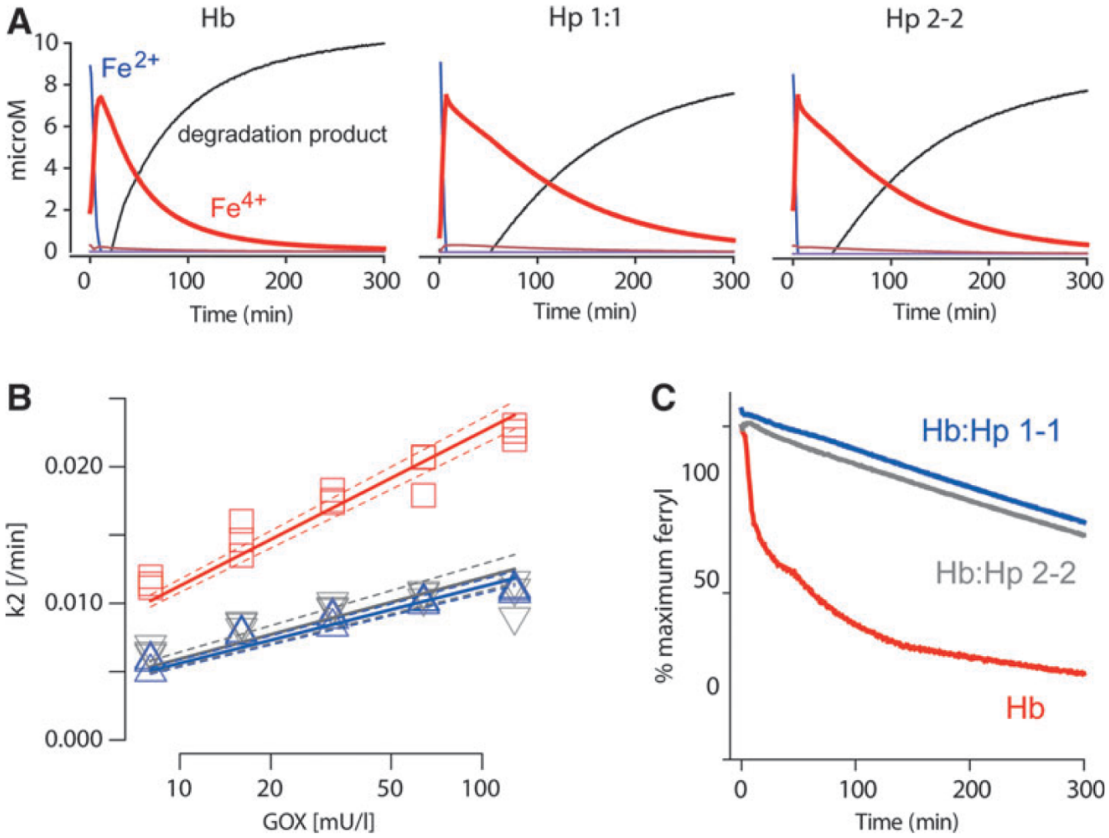

Figure 4: Hp stabilizes the Hb ferryl state. (A) oxy-Hb (12.5µM) with or without Haptoglobin of both phenotypes was incubated with 40mU of glucose-oxidase (GOX) as a source of continuous H_2O_2 at 37°C. The absorbance over a wavelength range of 300-700nm was recorded during 5h. The amounts of different Hb reaction products were extracted by spectral deconvolution. While Hp1-1 and Hp2-2 complexes of Hb indicate identical reaction patterns over time, free Hb shows a more rapid decline of ferryl-Hb concentrations (red line) with a concurrend more rapid increase of structurally nondefined porphyrin degratdation products (black line). (B) Ferryl decay rates (k_2) over a range of GOX concentrations. Experimental design and readout was shown in (A). Red: unbound Hb, blue: Hb:Hp1-1, gray: Hb:Hp2-2. The plotted line represents a linear regression ± 95% confidence interval (N=3 experiments). (C) identical experiments were repeated with 200nM of bovine catalase added after the first 12min. The recordings demonstrate that, in the presence of both Hp phenotypes, ferryl-Hb is much more stable compared to free Hb. *(This figure is part of publication 1)*

endothelial response observed without cross-linking. Finally, we could show that Hb bound to Hp also shows no precipitation and no endothelial toxicity. Hp is thus able to attenuate this principal pathway of peroxidative endothelial Hb toxicity.

By binding to Hp, Hb is structurally stabilized in such a way that the instable high-oxidation state ferryl-Hb ($Fe^{4+}=O^{2-}$) is concealed and the porphyrin radical produced during ferryl formation can be scavenged within the protein complex. This prevents the malicious Hb globin oxidation and denaturation and also prevents the formation of the toxic globin denaturation product. We could measure ferryl decay as an indicator for radical externalization and degradation by kinetic spectrophotometry and spectral deconvolution. We determined the ferryl decay rate and found that Hp1-1 as well as Hp2-2 both are able to

significantly increase ferryl stability (Figure 4)[1]. This mechanistically explains the attenuation of formation of denatured globin products by Hb peroxidation by Hp.

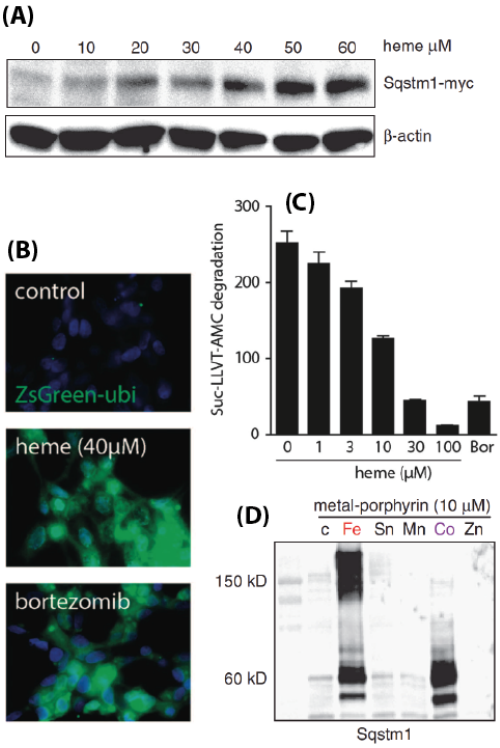

Figure 5 Direct heme toxicity: (A) Anti-myc western blot of Sqstm1-myc expressed under a constitutive CMV promoter in stably transfected RAW-264 cells to a range of heme concentrations. Sqstm1-myc accumulates with increasing heme concentration. (B) Effect of heme and proteasome inhibitor bortezomib (100pM) treatment for 6h on the accumulation of an ubiquitin-tagged fluorescent protein (ZsGreen) expressed in HEK-293 cells. Proteasomal degradation is inhibited by heme and bortezomib (positive control). (C) Enzymatic activity of purified 26S proteasome with increasing concentrations of heme as assessed by monitoring the release of fluorescent AMC from the proteasome substrate peptide Suc-LLVT-AMC. Bortezomib, a proteasome inhibitor, (Bor) at a concentration of 100pM was used as a positive control. (D) Hmox1 (-/-) MEFs were incubated with different metal protoporphyrins (PP) for 18h. Expression of Sqstm1 protein was probed by western blot. Accumulation of Sqstm1 and high-molecular-weight Sqstm1-protein aggregates in cells treated with FePP and CoPP can be observed. However, CoPP has not oxidative activity whereas FePP shows not only proteasomal inhibition but also protein oxidation followed by aggregation. *(This figure is part of publication 3)*

By oxidizing oxyHb (Fe^{2+}) to metHb (Fe^{3+}) also heme binding is altered. While heme is tightly bound to the globin in oxyHb, in metHb heme is only loosely bound in the heme pocket and can easily be released. By measuring the transfer to Hemopexin (Hx), an abundant plasma protein with very high heme affinity by spectrophotometry, we could determine the heme transfer-rate as $3.51s^{-1}$. By binding to Hp, heme binding of metHb is strongly improved and transfer-rates decrease to $0.05s^{-1}$ for Hp1-1 and $0.07s^{-1}$ for Hp2-2, respectively. Also during globin oxidation by oxidating Hb with H_2O_2, heme is ultimately released.

Free heme toxicity

Free heme itself shows direct cellular toxicity[3]. We could identify two synergistic mechanisms of heme toxicity (Figure 5): First, heme inhibits the proteasome, the intracellular garbage collection and degradation system. Proteins are marked for degradation by ubiquitination. We could show that heme causes accumulation of ubiquitinated proteins as well as the ubiquitin adaptor protein sequestosome (Sqstm1). Heme inhibits the enzymatic activity of the 26S-proteasome in a dose-dependent manner by direct binding to this protein. Second, heme directly oxidizes proteins and causes covalent protein-protein crosslinking and protein-lipid crosslinking products. These oxidative protein degradation products are potentially cytotoxic and are normally disposed by the ubiquitin-

sequestosome-proteasome pathway. Due to blockage of this pathway, degradation of these products is impaired, leading to a synergistic effect of the two mechanisms of direct heme toxicity.

Hb toxicity caused by lipid peroxidation

Because endothelial Hb toxicity may not be dependent of an external oxidator, we designed a model that allowed us to observe cellular effects in parallel to exact biochemical measurements in the absence of an exogenous oxidant such as H_2O_2. In the described previous works, we repeatedly identified oxidized lipoprotein as potential mediators of Hb toxicity after addition of human low-density lipoprotein (LDL). However, human LDL is not a homogenous compound, it contains β-carotene, a strong lipophilic antioxidant, in inter- and intraindividually varying amounts and also the lipid composition is not constant. Therefore, it is not feasible for systematic experimentation. As a replacement, we used a reconstituted lipoprotein (rLP) labored from apoA_1-lipoprotein purified from human plasma in an artificial liposome together with soy bean lecithin (CSL-111[20,21]). This particle has similar physical and chemical properties to human lipoproteins but is biochemically well defined, contains no antioxidants and thus enables reproducible results.

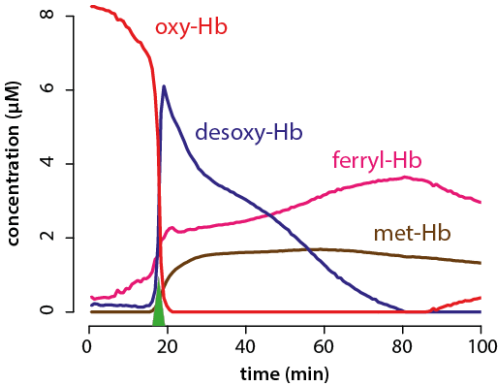

Figure 6: Time course of different Hb oxygenation and oxidation states during the reaction of oxyHb with rLP (rLP 0.5g/l, Hb 10µM, PBS pH 7.4, 37°C) as observed by spectrophotometry and spectral deconvolution. The Hb desaturation time (t_{des}) is indicated by the green triangle. *(This figure is part of publication 4)*

We biochemically characterized our rLP model by spectrophotometry. If oxyHb (10µM) and rLP (0.5g/l) are mixed, after a defined and well reproducible time, a sudden desaturation of oxyHb occurs after about t_{des}=18min at 37°C (Figure 6). After desaturation the build up of oxidized Hb species can be observed and lipid oxidation products can be detected. To characterize lipid oxidation we used a TBARS-Assay (thiobarbituric acid reactive substances) for quantification and mass spectrometry for identification as well as 4-HNE (4-hydroxynonenal) adducts as independent verification. We then used our system with endothelial cells in an ECIS (electrical cell substrate impendance sensing) setup and observed endothelial toxicity (defined as 50% decline in transendothelial resistance) after a few hours.

We found, that t_{des} can be shortened by replacing oxyHb by more readily available heme such as in the form of metHb or even by directly adding free heme to the system. We also found that t_{des} changed reciprocal to the starting heme concentration. We further found that antioxidants could slow down or even inhibit the reaction, however this effect was most pronounced if a lipophilic antioxidant such as butylated hydroxytoluene was used instead of a hydrophilic species such as ascorbic acid. This observation lead to the hypothesis, that free heme, a lipophilic molecule, has to transfer into the rLP compartment where it triggers a radicalic lipid oxidation in form of a self-enforcing chain reaction via heme-catalized formation of lipid peroxides: While without heme, lipid-autoperoxidation is very slow, (virtually a zero-order kinetic only dependent of the starting concentration of lipid radicals which is usually very low), heme accelerates the peroxidation exponentially to a chain reaction because it can generate two new lipid radicals out of one lipid peroxide. This also explains why free heme or metHb show a much faster t_{des} than oxyHb: While heme in oxyHb is tightly bound inside the heme pocket, metHb only loosely captures heme inside the pocket making it readily available. OxyHb has to auto-oxidate to metHb in order to start the reaction which is rate-limiting step in this model.

We used this rLP model to extensively study the role of Hp and Hx as a protective system against Hb-driven oxidative damage. Hp binds irreversibly to Hb, forming the very stable Hb:Hp complex. Although susceptibility to oxidation from ferrous to ferric state is not significantly altered by Hp, ferric Hb:Hp encapsulates heme very firmly and virtually no heme is released even in the oxidated metHb-state[2]. This protective mechanism only works, however, if all available Hb is complexed to Hp; otherwise some heme will always be released and begin its vitious cycle. Hp thus protects in a spatial way. Hx on the contrary does not bind to Hb at all. Because of its very high affinity to heme, it will bind all heme released from metHb as long as native apoHx is available. Thus, Hx can prevent Hb toxicity at already low substoechiometric levels relative to Hb, although only for a limited time (the protection is temporary). This mechanism explains the molecular basis for the observation that during hemolysis the first line of defense is Hp, which is readily depleted, followed by the second line of defense Hx, which only decreases after Hp has been exhausted.

To demonstrate the spatial protection of Hp and the temporal protection of Hx, we used the rLP system in parallel to measure t_{des} as a marker of the biochemical effect of heme-catalyzed lipid peroxidation and loss of transendothelial resistance as a surrogate of endothelial toxicity. We could show, that hemopexin prolongs endothelial survival as well as t_{des} directly proportional to the molar ratio to Hb (Figure 7). This is explained by the heme-capturing mechanism of endothelial protection by Hx. While oxyHb slowly auto-oxidizes to

metHb, Hx immediately extracts heme out of newly formed metHb and prevents transfer into rLP, halting the reaction for a limited period of time if used in sub-stoichiometric ratio to

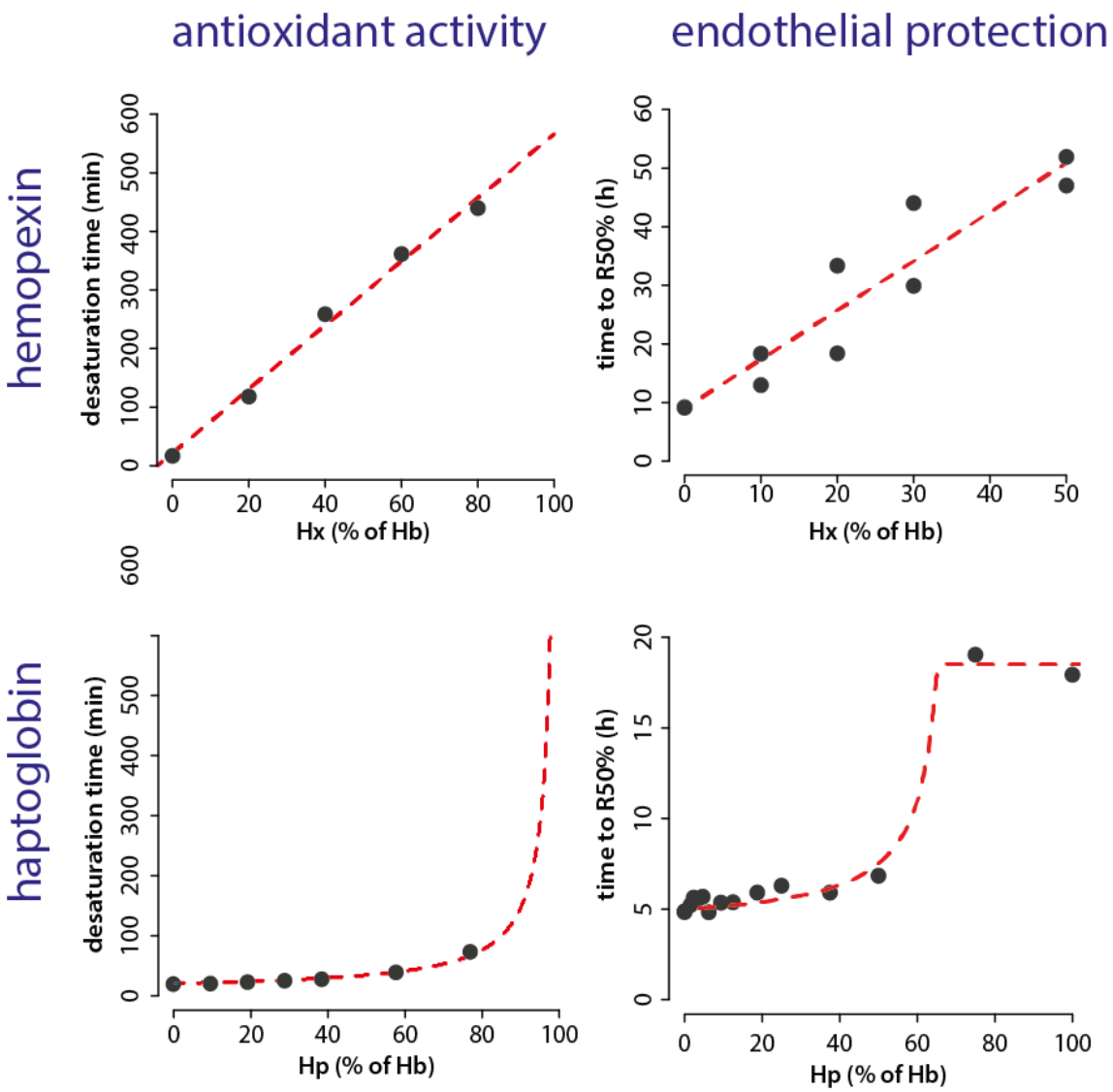

Figure 7: Stoichiometric requirements for protection by Hp and Hx. 10µM 10µM oxyHb where reacted with 0.5g/l rLP in the presence of Hx (upper graphs) or Hp (lower graphs) over a range of Hb binding protein concentrations between 0% and 100% binding capacity relative to heme. OxyHb concentrations was measured by spectrophotometry and spectral deconvotlution. In the left graphs, time to Hb desaturation of each sample is plotted against Hb binding protein capacity of Hx and Hp, respectively. Identical reactions were performed in HUVEC cell culture and transendothelial electrical resistance was measured by ECIS. In the right panels, the time to loss fo 50% of the resistance was plotted against the Hb binding capacity of Hx and Hp, respectively. *(This figure is (a modified) part of publication 4)*

Hb. In contrast to Hx, Hp shows virtually no protective effect if used in sub-stoichiometric amounts relative to Hb, explained by the spatial protection mechanism: Only of every Hb molecule is bound to Hp a heme release can be certainly inhibited. And only in this condition transfer of heme into rLP and subsequent firing up of the heme-catalized lipid-peroxidation can be prevented.

Conclusions and Outlook

We have identified oxidation of lipoproteins and subsequent endothelial damage as a major pathway of Hb toxicity. Heme release from oxidized Hb, followed by transfer of Heme into liposomes and induction of a radicalic lipid oxidation cycle producing cytotoxic lipid degradation products are the key steps of endothelial Hb toxicity. Additional to toxicity via lipid degradation products, we could show that heme also has a direct cytotoxic activity by inhibiting cellular degraded protein disposal via the proteasome. This effect is potentiated by the ability of heme to directly oxidize proteins; this leads to an intracellular accumulation of degraded, oxidized proteins by both, increased production and impaired removal.

We have extensively studied the protective activity of the natural Hb scavenger Hp. We could show, that both phenotypes Hp1-1 and Hp2-2 inhibit oxidative Hb degradation and heme release extremely effective. Further we could show, that both phenotypes exert this protective effect not only in biochemical assays but also *in vitro* and *in vivo*. Because Hp not only binds to Hb with high affinity but also structurally stabilizes the complex in such a way that heme release, protein oxidation (but not heme oxidation) are inhibited, heme is never released from Hp bound Hb and thus the toxicity described above can not evolve.

Although Hx has been identified as a protein being consumed during hemolysis and also as a possible protective agent in Hb toxicity, the significance of this effect could not be coherently explained since Hx can only bind to free heme but not directly to Hb. By identifying heme release as the rate limiting and crucial step in Hb toxicity, we where able to integrate Hx into the model of Hb toxicity and explain its protective role. Because Hx has an extremely high affinity to free heme, this protein is able to capture heme being released directly after it leaves Hb and thus preventing transfer into the liposome. Since exactly this transfer is the crucial step for the development Hb toxicity, Hx is an extremely potent and effective inhibitor thereof.

The different modes of protection of Hp and Hx explain their sequential decline during hemoglobinemia: As long as less free Hb is available than Hp is present, Hp binds all Hb, inhibits heme release and facilitates sequestration of Hb by $CD163^{+}$ macrophages. The situation changes as soon as the available Hp is saturated with Hb and even more Hb is released. In this situation Hx acts as a backup system by directly capturing released heme. The advantage of directly capturing free heme is, that only substoichiometric amounts of Hx are necessary in relation to free Hb since only the heme released from Hb has to be covered by Hx and not every Hb molecule as it is the case for Hp.

With this cumulative work, we have further explained the mechanisms of endothelial Hb toxicity via heme release and heme-mediated lipid oxidation. We have also shown and explained the protective roles and effects of the plasma proteins Hp and Hx. These results are however limited to endothelial toxicity; the mechanisms Hb toxicity on other tissues and organs such as the kidney remain to be explored, although a heme-mediated effect could be postulated based upon our data. Also the significance of heme mediated endothelial Hb toxicity in the overall context of the systemic toxicity of Hb, which for instance also includes vasoconstriction by NO depletion, is to be demonstrated in further *in vivo* studies.

Because Hp as well as Hx are natural plasma proteins, they are readily available since efficient plasma fractionation methods exist for both; and due to their intrinsic nature also toxicity or anaphylactic reactions are unlikely. Subsequent in-vivo and later clinical studies are necessary to confirm the protective potency of Hp and Hx with further data based on the fundamental biochemical and cell biological mechanics identified within this thesis.

References

Remark: The first four references are also listed under the table of contents.

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Authorship contribution of Jeremy Werner Deuel (JWD) to the publications that are part of the cumulative PhD thesis

Publication 1: Human Hp1-1 and Hp2-2 phenotype-specific haptoglobin therapeutics are both effective in vitro and in guinea pigs to attenuate hemoglobin toxicity.

JWD described the spectrophotometric method to simultaneously measure the different oxidation states of Hb and thus have measured the stability of the ferryl state of Hb and Hb:Hp complex (Figure 7) and modeled the decay rate according to the description in the methods section ("Spectral analyses of Hb H_2O_2 reactions"). He further developed the method to spectrophotometrically determine heme release from metHb and metHb:Hp complex (Figure 8B) and has performed the other spectrophotometric studies depicted in Figure 8A, C and D. JWD also developed the metHb decay model and thus quantified metHb decay resulting from heme transfer to hemopexin as shown in Table 1 and in the methods section ("Hb.Hpx heme transfer").

JWD co-first author together with Miriam Lipiski of this paper. He developed the methods and performed the experiments and analysis for the biochemical characterization of Hb, Hb:Hp1-1 and Hb:Hp2-2 complexes.

Publication 2: Mechanisms of haptoglobin protection against hemoglobin peroxidation triggered endothelial damage.

JWD quantified precipitation of different Hb by spectrophotometry (Fig 2A) under continuous oxidation by H_2O_2 produced by glucose oxidase. JWD developed a spectrophotometric method to absolutely quantify different Hb oxidation species in real-time during a reaction of Hb with H_2O_2 as shown in Figure 3A, B and C, thus making it possible to monitor and characterize the different Hb oxidation state and measure the stability of the ferryl state.

JWD is co-first author, together with Christian A. Schaer, of this paper. JWD developed the method to absolutely quantify the different Hb reaction species (oxyHb, desoxyHb, metHb and ferrylHb as well as degradation products) in real-time by spectrophotometry in the visible range by fitting extinction curves of the different species using the non-negative least squares algorithm. This allowed to develop the biochemical foundation of this paper and thus—together with the cell biology data—identify the toxic and non-toxic metabolites of Hb peroxidation.

Publication 3: Proteasome inhibition and oxidative reactions disrupt cellular homeostasis during heme stress.

RAW-264 cells were stably transfected by JWD with sqstm1-myc expressed under a constitutive CMV promoter and measured SQSM1 retention after incubation with heme to prove that not only induction but also impaired degradation of sequestosome is an effect of heme toxicity (Figure 3G) as well of cobalt-protoporphyrin (Fig 7B). He also measured retention of ZsGreen-Ubiquitin in HEK-293 cells after incubation with heme and bortezomib, again showing that proteasome inhibition and thus accumulation of ubiquitin tagged proteins is a heme effect (Figure 4B). A fluorescent proteasome activity assay using Suc-LLVT-AMC was developed and used by JWD, showing proteasomal inactivation by heme in a biochemical model (Figure 4D). The independence of the presence of an antioxidant for proteasomal inhibition (Figure 7G) was also demonstrated by him.

In summary, JWD contributed to the mechanistic proof that heme inhibits proteasomal activity and thus ubiquitinated proteins and sequestosome are retained by setting up a proteasome activity assay and a cellular model of sequestosome and ubiquitin retention.

Publication 4: Different target specificities of haptoglobin and hemopexin define their sequential role as a primary and backup protective system against hemoglobin toxicity.

JWD and Dominik J. Schaer designed the experiments and wrote this paper. JWD performed the experiments on cellular biology on vascular endothelial cells (Figures 1D, 2, 3D and 6B), but also performed most of the biochemical analyses. He measured Hb redox state during reaction with rLP (Fig 3A), verified the measurements by direct O_2 measurement and correlation with the spectrophotometric data (Fig 3B), and did all the studies involving Hb deoxygenation (Fig 5 and 6A) as well as TBARS quantification (Fig 7A). Together with Michele Puglia he analyzed the mass spectrometry data and thus identified oxidized lipids (Figures 4A and 4C). Also the explanatory models and the corresponding figures explaining our hypothesis and findings (Figure 1E, 3C and 7B) where designed by JWD.

This is the paper resulting from the experiments in conjunction with the hypotheses presented in the application to the MD-PhD-programs.